

Original citation:

Kowalczyk, Agnieszka, Price, Oliver R., van der Gast, Christopher J., Finnegan, Christopher J., van Egmond, Roger A., Schäfer, Hendrik and Bending, Gary D.. (2016) Spatial and temporal variability in the potential of river water biofilms to degrade p-nitrophenol. *Chemosphere*, 164. pp. 355-362.

Permanent WRAP URL:

<http://wrap.warwick.ac.uk/82235>

Copyright and reuse:

The Warwick Research Archive Portal (WRAP) makes this work by researchers of the University of Warwick available open access under the following conditions. Copyright © and all moral rights to the version of the paper presented here belong to the individual author(s) and/or other copyright owners. To the extent reasonable and practicable the material made available in WRAP has been checked for eligibility before being made available.

Copies of full items can be used for personal research or study, educational, or not-for-profit purposes without prior permission or charge. Provided that the authors, title and full bibliographic details are credited, a hyperlink and/or URL is given for the original metadata page and the content is not changed in any way.

Publisher's statement:

© 2016, Elsevier. Licensed under the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International <http://creativecommons.org/licenses/by-nc-nd/4.0/>

A note on versions:

The version presented here may differ from the published version or, version of record, if you wish to cite this item you are advised to consult the publisher's version. Please see the 'permanent WRAP URL' above for details on accessing the published version and note that access may require a subscription.

For more information, please contact the WRAP Team at: wrap@warwick.ac.uk

Spatial and temporal variability in the potential of river water biofilms to degrade p-nitrophenol

**Agnieszka Kowalczyk^{*1}, Oliver R. Price², Christopher J. van der Gast^{1,3},
Christopher J. Finnegan², Roger A. van Egmond², Hendrik Schäfer¹ and
5 Gary D. Bending¹**

*¹School of Life Sciences, Gibbet Hill Campus, University of Warwick,
Coventry, CV4 7AL, UK.*

*²Unilever, Safety and Environmental Assurance Centre, Sharnbrook,
10 Bedfordshire, MK44 1LQ, UK.*

³NERC Centre for Ecology and Hydrology, Wallingford, OX10 8BB, UK

* To whom correspondence may be addressed:

15 Dr Agnieszka Kowalczyk. Telephone: +44 (0) 7758572996. E-mail: AKowalcz@scj.com
Present address: SC Johnson, Frimley Green, Frimley, Camberley, Surrey, GU16 7AJ,
United Kingdom

20 **Abstract**

In order to predict the fate of chemicals in the environment, a range of regulatory tests are performed with microbial inocula collected from environmental compartments to investigate the potential for biodegradation. The abundance and distribution of microbes in the environment is affected by
25 a range of variables, hence diversity and biomass of inocula used in biodegradation tests can be highly variable in space and time. The use of artificial or natural biofilms in regulatory tests could enable more consistent microbial communities be used as inocula, in order to increase test consistency. We investigated spatial and temporal variation in composition,
30 biomass and chemical biodegradation potential of bacterial biofilms formed in river water. Sampling time and sampling location impacted the capacity of biofilms to degrade p-nitrophenol (PNP). Biofilm bacterial community structure varied across sampling times, but was not affected by sampling location. Degradation of PNP was associated with increased relative
35 abundance of *Pseudomonas syringae*. Partitioning of the bacterial metacommunity into core and satellite taxa revealed that the *P. syringae* could be either a satellite or core member of the community across sampling times, but this had no impact on PNP degradation. Quantitative PCR analysis of the *pnpA* gene showed that it was present in all samples
40 irrespective of their ability to degrade PNP. River biofilms showed seasonal variation in biomass, microbial community composition and PNP biodegradation potential, which resulted in inconsistent biodegradation test results. We discuss the results in the context of the mechanisms underlying variation in regulatory chemical degradation tests.

Key words

Biofilm; Metacommunity; Seasonality, Biodegradation; OECD tests;

Pseudomonas; Para-nitrophenol

1. Introduction

50 There is a vast diversity of chemicals in the environment, which arrive
from industry, agriculture, medical treatment and common household
products. Many of these chemicals have the potential to exert adverse
human and ecological health effects (Mnif et al. 2011, Jones et al. 2003,
Lapertot and Pulgarin, 2006). The persistence of chemicals entering the
55 environment is controlled by a range of biotic and abiotic processes, which
together determine environmental concentrations of the chemical and the
extent to which it is transformed to metabolites (Kowalczyk et al. 2014). For
most chemicals, biodegradation, mediated by microorganisms, is the main
factor controlling persistence. In order to predict chemical persistence the
60 Organisation for Economic Cooperation and Development (OECD) has
established a tiered series of biodegradation tests (Kowalczyk et al. 2014).

 The first tier ready biodegradability tests are intended as screening
approaches to gauge if a chemical is rapidly degraded, or has potential to
degrade, in the environment. These tests utilize a variety of environmental
65 materials, such as water and sediments as inocula (Doi et al. 1996;
Reuschenbach et al. 2003; Yuan et al. 2004). Guidelines provide flexibility for
the inoculum that can be used and its method and time of collection.
However, it is known that inoculum (i.e. microbial biomass) density is an
important factor affecting the outcome of chemical biodegradation tests
70 (Thouand et al. 1995; Godhead et al. 2014) and in particular, inconsistent
test results may reflect differences in inoculum quality because of temporal
and spatial variability of microbial community density and diversity within
environmental compartments (Courtes et al. 1995; Mezzanotte et al. 2005).

Microbial communities are subject to seasonal variability due to changes
75 in environmental conditions, which shape natural ecosystems and affect
processes occurring in environmental compartments (Bertram, et al. 2001;
Yunus and Nakagoshi, 2004). In particular, changes in river water volume
and flow rates (Naudin et al. 2001), temperature (LaPara et al. 2000), light
penetration (Romaní and Sabater, 1999) and suspended matter (Cébron et
80 al. 2007) can impact the composition of microbial communities inhabiting
river water and sediment (Hudon, 1997; Hunt and Parry, 1998; Midwood and
Chow-Fraser, 2012, LaPara et al. 2000). This can affect chemical
bioavailability and biodegradation *in situ*. For example, seasonal variations in
biodegradation of 2,4-dichlorophenoxyacetic acid (2,4-D) were reported by
85 Watson (1977) in river water and sediment, with maximum breakdown
observed during winter flood conditions, probably because of adsorption of
the chemical onto suspended sediment, where microbial activity was high
(Nesbit and Watson, 1980). Similarly, seasonal variation in degradation of
hexadecane, associated with changes in nutrient availability and biofilm
90 composition, was reported by Chénier et al. (2003).

As an alternative to the use of natural inoculum, there has been interest in
generating standardised biofilms for use in chemical biodegradation tests
(Kowalczyk et al. 2014). Currently, biofilms are used as inocula in some
OECD tests, such as the biodegradation simulation test guideline OECD 303
95 b (OECD, 2005), and bioreactors with biofilms comprising of defined
bacterial strains and mixtures have been applied to study the bioremediation
of a variety of chemicals, including chlorophenols (Puhakka et al. 1995; Kargi

and Eker, 2005), herbicides (Oh and Tuovinen, 1994) and azo dyes (Zhang et al. 1995).

100 In natural habitats, biofilms typically have high species richness (Sabater et al. 2007) and density (Singh et al. 2006), show resistance to toxic chemicals e.g. antimicrobial agents (Mah and O'Toole, 2001) and recover quickly from perturbation (Morin et al. 2007; Proia et al. 2010). Furthermore, biofilms support synergistic interactions within communities (Elias and Banin,
105 2012), provide microhabitat heterogeneity (Donlan, 2002), and the potential for genetic exchange (Schwartz et al. 2003), all of which can promote chemical biodegradation.

For these reasons, use of biofilms in biodegradation tests could provide a means of reducing the chance of common test failures described by
110 Thouand et al. (1995) and Godhead et al. (2014), by eliminating the 'biodegradation lottery' associated with low biomass in regulatory tests (Kowalczyk et al., 2014). However, to date, investigations of the potential to use biofilm inoculum in regulatory tests has centered on laboratory generated inoculum.

115 In recent studies we have used a variety of culture dependent and independent approaches, including DNA-stable isotope probing and functional gene markers to identify para-nitrophenol (PNP) degrading communities in a UK river, establishing that species of *Pseudomonas* were associated with degradation in samples taken between 2010 and 2013
120 (Kowalczyk et al. 2015). In the current study, we investigated temporal and spatial assembly of *in situ* river biofilm communities and relationships between community composition and biodegradation of PNP, addressing the

following questions: (1) how does time affect the composition and biodegradation potential of river water biofilms? (2) does spatial location
125 affect biofilm composition and degradation potential? and (3) does temporal and spatial variation in chemical biodegradation kinetics relate to dynamics of specific degraders within the biofilm community?

130 **2. Materials and methods**

2.1 Biofilm preparation

Biofilms were prepared in the River Dene, in Warwickshire, UK (52°11'58.54"N and 1°36'44.94"W). The location is a rural catchment and
135 there was no known previous exposure to industrial pollutants, including PNP. At each sampling time and location, Biofilms were generated on polysine glass slides (5.7 x 2.5 cm) (VWR, International) attached to a Hole Airbrick (21.0 x 13.5 x 5.0 cm) (Travis Perkins Trading Co. Ltd, UK) with cable ties (165 x 2.6 mm) (BHGS Ltd, UK). Each slide had a total surface
140 area of 28.5 cm². 11 slides were attached to each brick.

At each sampling time, two bricks with slides were placed on the bed of the River 200 m upstream, 200 m downstream and at the effluent discharge point of the Wellesbourne Wastewater Treatment Plant (WWTP) (52°11'58.54"N and 1°36'44.94"W). Bricks were left in the river for eight days
145 to allow colonisation of the slides by microorganisms and development of natural river water biofilms. Biofilms grown in this way were collected in November 2011, February 2012 and May 2012.

2.2 The biodegradation of PNP by river biofilms

150 At each sampling time, biofilms collected from each site were used to determine potential for PNP biodegradation. For each site, triplicate tests were set up, consisting of a sterile amber Duran Schott bottle (500 mL) (Fisher Scientific, UK) with 300 mL of sterile river water with 2 mg L⁻¹ PNP, to which were added three of the Polysine glass slides supporting river water
155 biofilms.

Sterile river water was prepared by autoclaving water samples collected downstream of the River Dene STP at 121°C, for 15 minutes, at 1.1 atm. A positive control consisted of sterile river water inoculated with the PNP-degrading isolate *Pseudomonas syringae* AKHD2 (Kowalczyk et al., 2015),
160 grown on LB medium with PNP (56 mg/L) for 48 hours at 25°C. Sterile river water was used as a negative control. Additionally for the February and May 2012 time points, non-sterile river water collected from the effluent location was used to compare degradation rates with the biofilm materials. PNP (dissolved in water) was added to biodegradation test bottles as a sole
165 source of carbon to a concentration of 2 mg L⁻¹. Bottles were incubated in a controlled environment room with an 18 hour light and six hour dark cycle at 20±2 °.

Every two days, a 1 mL aliquot of liquid was taken from each bottle and used to determine PNP concentration using HPLC, which comprised a
170 Hewlett Packard 1100 HPLC system with a LiChrosphere (5 µm) C-18 column (MerckMillipore, UK). The mobile phase was a mixture of water: methanol (HPLC grade, Fisher Scientific, UK) with a ratio of 40:60 (v/v) and

the flow rate was 0.50 mL/minute. Analysis was performed with UV detection at 254 nm, with column pressure of 115 bar and column temperature at 25 °C. The volume of the injected sample was 20 µL and the run time was 13 minutes. HPLC calibration was carried out using concentrations of 2-2000 µg/L of PNP standards.

Time to 50 % PNP-degradation (DT50) was calculated using the model of best fit to the biodegradation kinetics for each sample, as described by Rodriguez-Cruz et al, (2006). Least significant difference (LSD) was used to determine significant differences in DT50 between treatments using GenStat (13th edition, VSN International Ltd.).

2.3 Biofilm biomass

Extracellular polysaccharide (EPS) was measured as a proxy for biofilm biomass (Flemming et al. 2007; Underwood and Paterson, 1995). At each sampling time and location, biofilm was removed from 3 glass slides using a sterile nylon brush and pooled, as described by Deines et al. (2010). Soluble carbohydrate was determined by reaction with phenol and H₂SO₄ (Dubois et al. 1956) using glucose as a standard. LSD was used to determine significant differences between treatments.

2.4 DNA extraction from biofilms

Before incubation (T₀), after complete PNP biodegradation or when there was no biodegradation at the end of the incubation (T_{end}), biofilms were harvested from slides in each bottle as described above. The suspended

biofilm biomass was collected on a Millipore GVWP04700, (hydrophilic Durapore) polyvinylidene fluoride membrane filter (0.22 µm mesh, 47 mm diameter) using a vacuum filtration system. Membrane filters with concentrated biofilm biomass were folded and placed in the bead tubes of the Power Water DNA extraction kit (MoBio, UK), before DNA extraction according to the manufacturer's protocol. DNA was extracted from effluent location water samples using procedures described in Kowalczyk et al. (2015). The DNA concentration was measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific Fisher, UK).

2.5 Quantitative PCR analysis of *pnpA* gene.

Our earlier work (Kowalczyk et al. 2015) showed that p-nitrophenol degradation in the River Dene is mediated through the Gram-negative degradation pathway, involving the gene *pnpA*. Quantitative PCR was used to determine the number of *pnpA* genes in DNA extracted from biofilm and effluent water samples prior to and following degradation of p-nitrophenol, using approaches described in Kowalczyk et al. (2015). LSD was used to determine significant differences between treatments.

2.6. Microbial community structure

16S rRNA Terminal Restriction Fragment Length Polymorphism (TRFLP) was performed to determine bacterial community structure in T_0 and T_{end} biofilms and effluent water samples. Procedures described in Kowalczyk et al. (2015) were used for PCR, restriction digestion and analysis.

2.7 Bacterial community analyses

225 Bray Curtis dissimilarity matrices of bacterial communities were generated from TRFLP data, and visualized using non-metric multidimensional scaling (NMDS), with Analysis of Similarities (ANOSIM) used to determine significance of differences between samples (Hilton et al. 2013). These analyses were performed using Primer 6 software V10.1.12 (PRIMER-E Ltd. 230 UK). TRF 245 nt was identified in previous work as a specific marker for *Pseudomonas syringae*, which was linked to degradation of PNP in parallel studies over the same sampling period (Kowalczyk et al. 2015). Relative abundance of TRF 245 nt as a percentage of total fluorescence intensity was determined, and Analysis of Variance used to compare differences across 235 sampling locations and times.

2.8 Analyses of core and satellite taxa

2.8.1 Analysis of the local community

Each biofilm bacterial TRFLP profile was treated as a local community. 240 Rank-abundance plots were used to determine differences in bacterial local community structure for each sampling site and incubation time point (T_0 and T_{end}). Rank-abundance plots were constructed using the relative abundance of each TRF (taxon) The rank-abundance plots were visualized by plotting the taxa rank order on the x-axis against relative abundance (\log_{10} 245 transformed) on the y-axis. For each plot a linear regression model was fitted, represented by the equation, $\log_{10}y = a + bx$, where a is the intercept and b is the slope of the plot. The slope (b) was used as a descriptive

statistic to compare community structure (Ager et al. 2010). Linear regressions (r^2) were calculated using Microsoft Excel, and one-way ANOVA, using GenStat13th edition, was used to assess significance between sampling times and locations.

2.8.2 Analysis of the metacommunity

The two-group core-satellite modeling approach (van der Gast et al. 2011) was applied to the bacterial metacommunities to (1) establish whether the PNP-degrading *Pseudomonas syringae* (i.e. TRF 245 nt) was a core or satellite component, (2) determine whether distribution and persistence of *P. syringae* was affected by sampling date, and (3) investigate how relative abundance and distribution of *P. syringae* correlated with PNP biodegradation.

The TRFs in each biofilm metacommunity were divided into core and satellite groups by decomposing the overall distribution using the ratio of variance to the mean abundance for each bacterial taxon (van der Gast et al. 2011). The variance to mean ratio, or index of dispersion, is an index used to model whether species follow a Poisson distribution, falling between 2.5% and 97.5% confidence limits of the χ^2 distribution. Bacterial TRFs that occurred only once in TRFLP profiles were excluded from this analysis, as their dispersion in space would have no variance. Poisson distribution tests were carried out according to the method described by Krebs (1999). Rank-abundance plots with core and satellite taxa were visualized by plotting the taxa rank order on the x-axis against mean % relative abundance on the y-axis with \log_{10} scale (Rogers et al. 2013). An Excel macroprogram was applied to compare the slopes of rank-abundance plots with core and

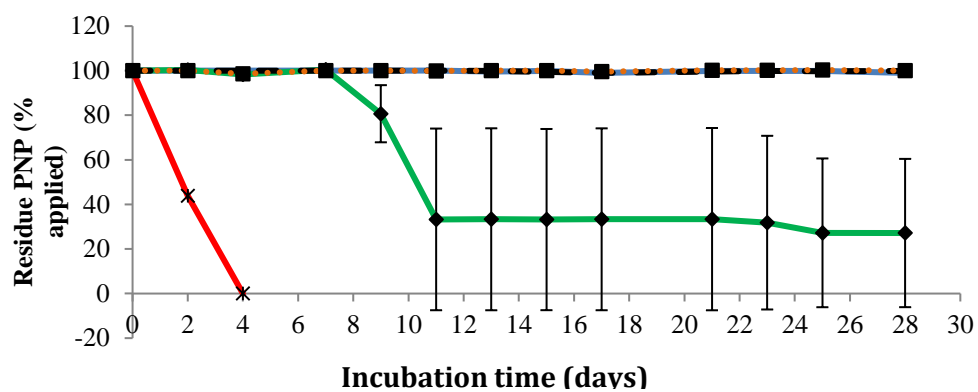
satellite species between metacommunities to determine the significance of differences in metacommunity structure between sampling dates and incubation time points. Regression analysis, coefficients of determination (r^2), residuals and significance (p) were calculated using Minitab software (version 15, Minitab, University Park, PA, USA).

3. Results

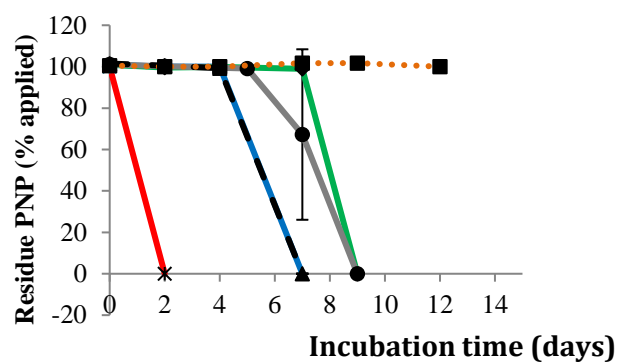
3.1 The biodegradation of PNP

DT₅₀ ranged between 5.5 and 16 days across sampling times and locations. No PNP biodegradation (Figure 1) was observed for upstream and downstream river water biofilms collected in November 2011, while two out of three replicates of effluent biofilm completed PNP biodegradation within nine days (Figure 1). There was a significant difference in DT₅₀ between effluent biofilms collected in November 2011 (16.11 days) and February 2012 (8.2 days) (Table 1). There were no significant differences in DT₅₀ between sampling locations in February or May 2012, except effluent water, for which DT₅₀ was significantly faster (6.2 days) in May relative to February 2012 (7.3 days).

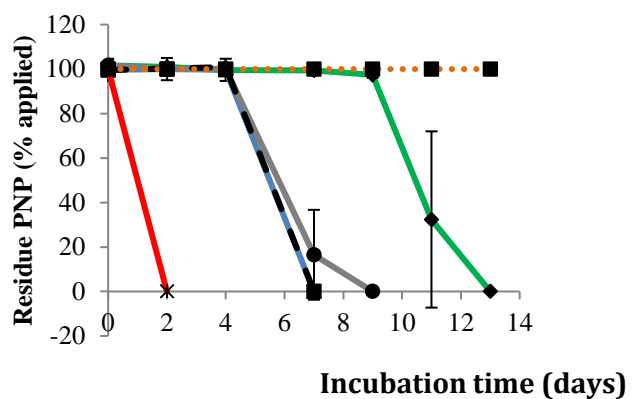
a. November 2011



295 **b. February 2012**



c. May 2012



■ Upstream biofilm ◆ Effluent biofilm ● Effluent river water
 ▲ Downstream biofilm * Positive control ● Negative control

300 Fig. 1. The biodegradation of PNP in biofilms and river water.

Inoculum collected in a, November 2011; b, February 2012; c, May 2012.

Average values from triplicate samples. Error bars show standard error of the mean (S.E.M.), n=3.

305 Table 1. Time to 50 % biodegradation (DT₅₀) of PNP using biofilm and river water inoculum.

Date	Location	DT ₅₀
November 2011	Upstream	ND
	Effluent	16.11(±5.95) ^a
	Downstream	ND
February 2012	Upstream	5.50(±0.00) ^{bc}
	Effluent	8.20(±0.00) ^{bc}
	Downstream	5.50(±0.00) ^{bc}
	Effluent water	7.30(±0.89) ^c
May 2012	Upstream	5.63(±0.03) ^{bc}
	Effluent	10.83(±0.63) ^{ba}
	Downstream	5.70(±0.03) ^{bc}
	Effluent water	6.20(±0.40) ^b

Treatments with different letters are significantly different (P<0.05); ± standard error of the mean (S.E.M.); n=3.

310 **3.2 Biofilm biomass**

Sampling date and location were found to significantly affect the amount of biofilm that had developed on glass slides (Table 2). Effluent biofilm had significantly lower biomass than upstream and downstream biofilms at all-time points. Biofilms collected in May 2012 had up to twice as much biomass
315 as those collected in November 2011 and February 2012. There was no significant correlation between DT50 for PNP biodegradation and the amount of biofilm biomass on the glass slides at T_0 .

Table 2. Biofilm biomass concentrations.

320

Date	Sampling site	EPS* as glucose equivalent (µg/L)
November 2011	upstream	251 ^a (± 3.80)
	effluent	73.2 ^b (± 0.63)
	downstream	252 ^a (± 5.70)
February 2012	upstream	227 ^c (± 2.21)
	effluent	173 ^d (± 9.17)
	downstream	134 ^e (± 1.90)
May 2012	upstream	668 ^f (± 1.26)
	effluent	261 ^a (± 0.95)
	downstream	635 ^g (± 0.63)

Treatments with different letters are significantly different ($P < 0.05$); \pm standard error of the mean (S.E.M.), $n=3$,

*EPS: extracellular polysaccharide.

325

3.3 Biofilm and river water bacterial community

3.3.1 NMDS analysis of TRFLP data

Bacterial TRFLP profiles of river water and river biofilm bacterial profiles
 330 collected on different sampling dates and locations were compared before
 and following degradation of PNP. NMDS and ANOSIM (Figure 2) revealed
 that the bacterial community structure of river water and biofilms clustered by
 sampling date with significantly different (20 % similarity) profiles for each
 season. There was a significant shift ($p < 0.001$) in the community from T_0 to
 335 post-PNP degradation (T_{end}), the nature of which was specific to each
 sampling time. Location of biofilm sampling site had no significant effect on
 bacterial community profiles and upstream, downstream and effluent biofilms
 clustered together by sampling time.

340

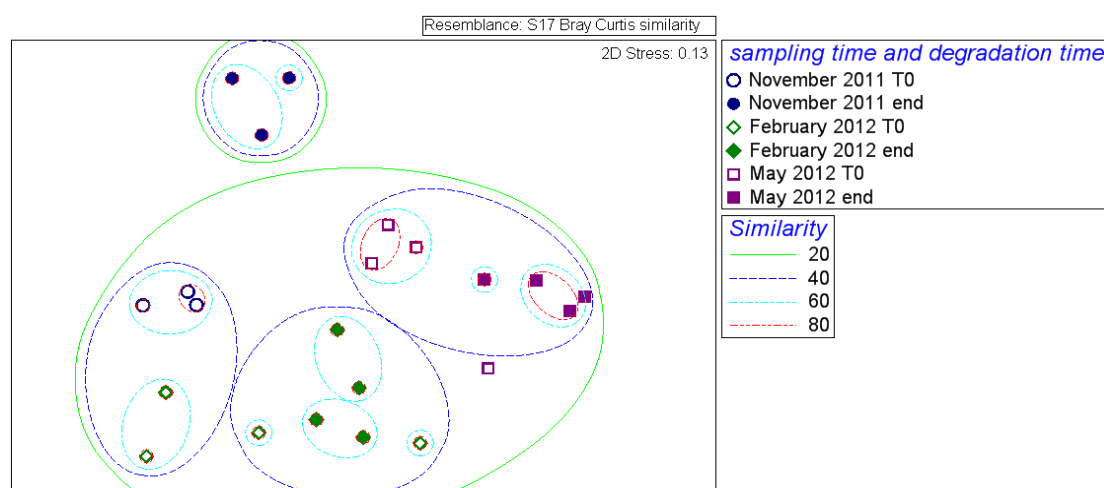
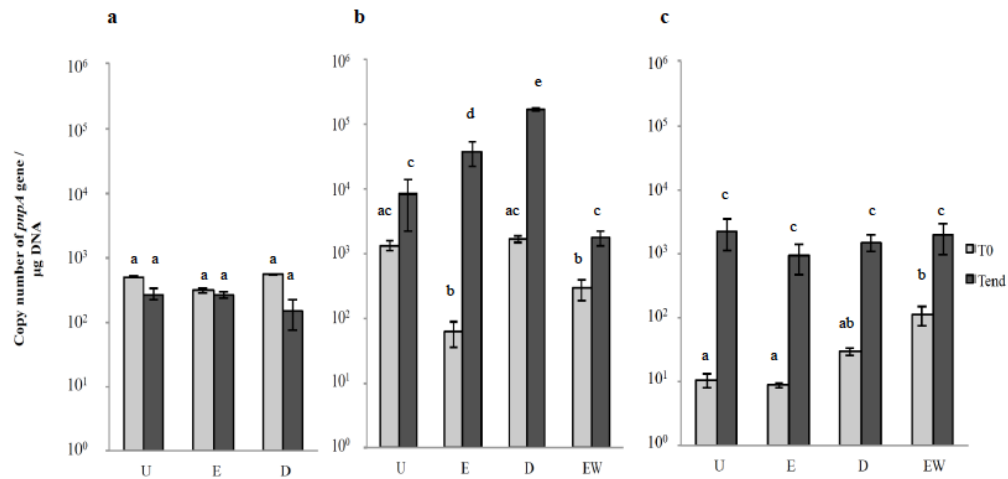


Fig 2. Non-metric multidimensional scaling (NMDS) of bacterial community structure prior to and following degradation of PNP across sampling times. Each symbol shows the mean of 3 replicates. T_0 , freshly collected
345 biofilm/river water; T_{end} , biofilm/river water after incubation/complete PNP biodegradation.

3.3.2. Quantitative PCR analysis of the *pnpA* gene

There were no significant differences in the copy number of *pnpA* at T_0 or
350 T_{end} between biofilms collected from different sampling points in November (Figure 3), in which degradation occurred only in which 2 of the 3 effluent replicates. However, with the exception of the upstream location in February 2012, there were significant differences in the number of *pnpA* genes between T_0 and T_{end} biofilms and effluent river water collected in February
355 and May, with a 10-100 fold increase following complete PNP degradation. No correlation was found between *pnpA* gene copy numbers and the number of TRFs detected in biofilms.



360

Fig 3. Copy number of *pnpA* genes prior to and following PNP degradation, across sampling locations and times.

Figure legend: a, November 2011; b, February 2012; c, May 2012, U, biofilm upstream; E, effluent biofilm; D, downstream biofilm; EW, effluent river water.

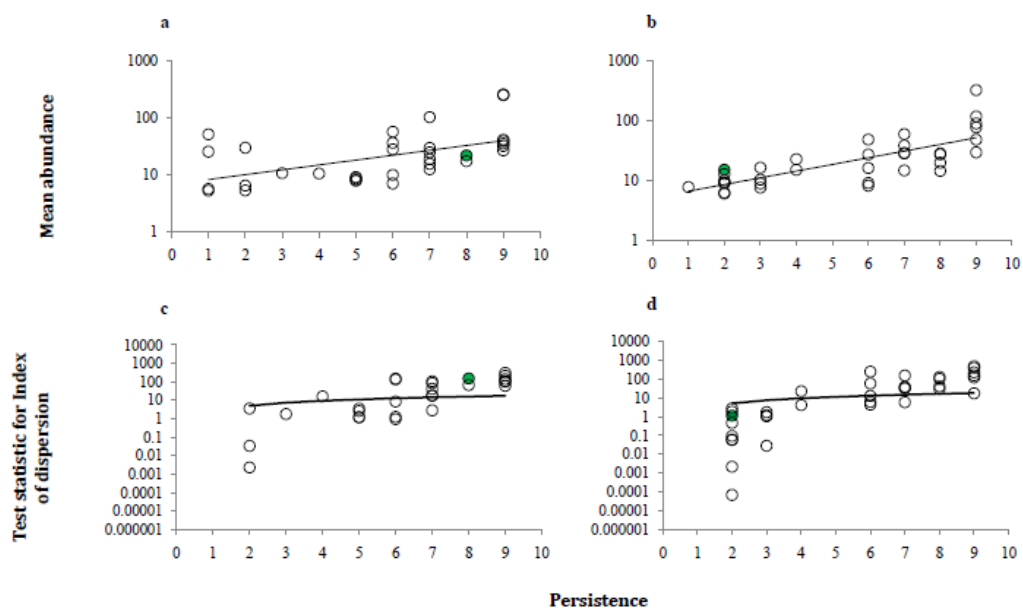
365 Treatments with different letters are significantly different ($P < 0.05$); error bars show \pm standard error of the mean (S.E.M.), $n=3$.

3.4 Core and satellite taxa

370 3.4.1 Biofilm metacommunity structure, distribution and persistence of *Pseudomonas syringae*

After dispersion was plotted against the persistence of taxa for biofilm metacommunities before and after degradation (SI Figure 1-3 a and b), Poisson distributions were fitted to identify core and satellite taxa (SI Figure 375 1-3 c and d). Since there was no significant difference in community composition by sampling location, upstream, downstream and effluent T_0 or T_{end} biofilms were pooled together by sampling time for this analysis. The taxa which fell below the 2.5% confidence limit line were identified as

randomly distributed satellite species. Taxa above the 2.5% confidence limit
 380 line were considered non-randomly distributed core species. It appeared that
P. syringae was a core taxon at T₀ in the November and February biofilm
 metacommunities but was a satellite taxon in the May T₀ metacommunity
 (Fig 4). After the biodegradation of PNP (T_{end}) *P. syringae* remained a core
 taxon in February 2012, but became the most abundant taxon, and similarly
 385 in May it increased enormously in relative abundance following
 biodegradation, to become the second highest taxon by relative abundance.
 In November 2011, the persistence of *P. syringae* decreased after incubation
 with PNP and shifted from being a core to a satellite taxon.



390

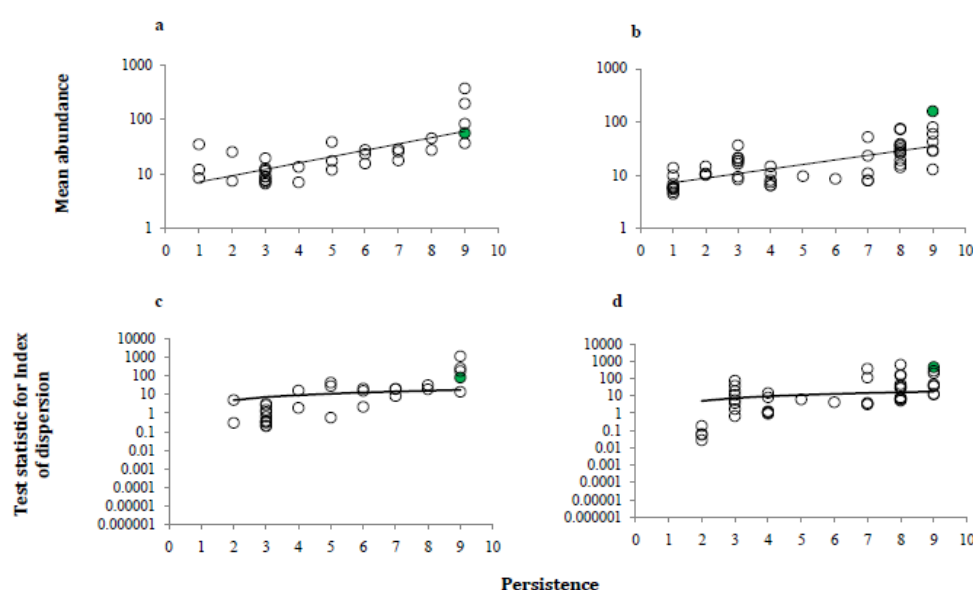
Supplement Fig. 1. Distribution and dispersal of bacterial taxa in biofilm communities in November 2011.

The number of biofilm TRFLP profiles for which each bacterial TRF (taxa) was observed, plotted against the mean abundance (log₁₀ scale) across all
 395 TRFLP profiles a, before incubation (T₀) ($r^2 = 0.29$; $F_{1,32} = 12.57$; $P < 0.0001$),

and b, after complete PNP biodegradation (T_{end}) ($r^2 = 0.60$; $F_{1,35}=51.57$; $P<0.0001$). c, d random and non-random dispersal of TRFs from biofilm TRFLP profiles before and following PNP degradation respectively (*P. syringae* highlighted in green). Dispersal visualised by decomposing the

400 overall distribution using an index of dispersion based on the ratio of variance to the mean abundance for each bacterial TRF from analysed 9 TRFLP profiles. The line depicts the 2.5% confidence limit for the χ^2 distribution. The 97.5% confidence limit was not plotted, as no taxon fell below that line.

405

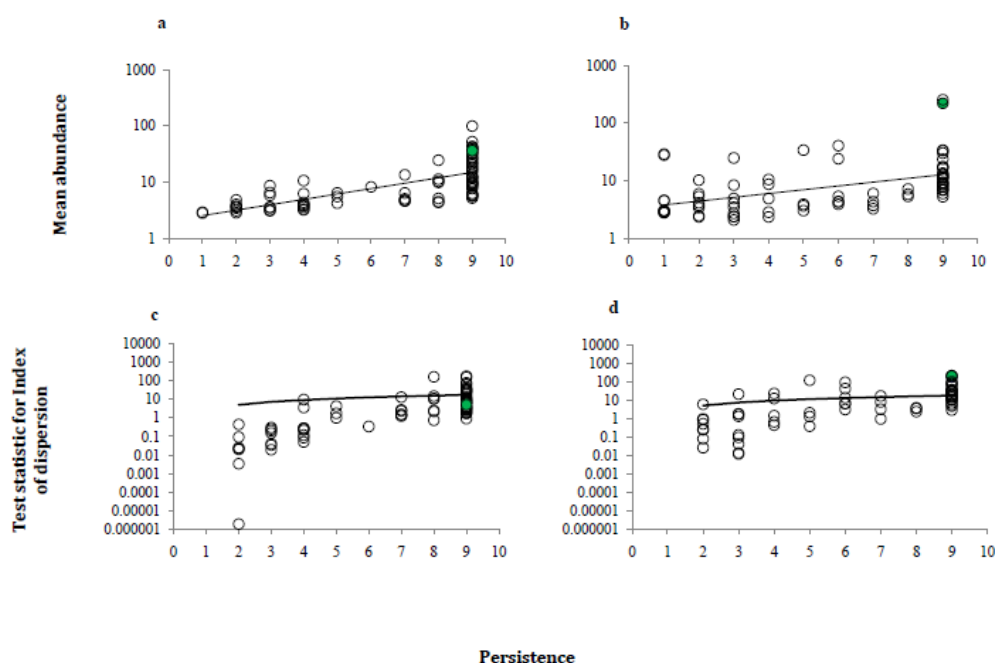


Supplement Fig 2. Distribution and dispersal of bacterial taxa in biofilm communities in February 2012.

The number of biofilm TRFLP profiles for which each bacterial TRF (taxa)

410 was observed, plotted against the mean abundance (log₁₀ scale) across all TRFLP profiles a, before incubation (T_0) ($r^2 = 0.55$; $F_{1,32}=37.52$; $P<0.0001$), and b, after complete PNP biodegradation (T_{end}) ($r^2 = 0.51$; $F_{1,54}=54.91$;

P<0.0001). c, d random and non-random dispersal of TRFs from biofilm TRFLP profiles before and following PNP degradation respectively (*P. syringae* highlighted in green). Dispersal visualised by decomposing the overall distribution using an index of dispersion based on the ratio of variance to the mean abundance for each bacterial TRF from analysed 9 TRFLP profiles. The line depicts the 2.5% confidence limit for the χ^2 distribution. The 97.5% confidence limit was not plotted, as no taxon fell below that line.



Supplement Fig 3. Distribution and dispersal of bacterial taxa in biofilm communities in May 2012.

The number of biofilm TRFLP profiles for which each bacterial TRF (taxa) was observed, plotted against the mean abundance (log₁₀ scale) across all TRFLP profiles a, before incubation (T_0) ($r^2 = 0.50$; $F_{1,80}=78.10$; $P<0.0001$),

and b, after complete PNP biodegradation (T_{end}) ($r^2 = 0.23$; $F_{1,76} = 22.04$;
 430 $P < 0.0001$). c, d random and non-random dispersal of TRFs from biofilm
 TRFLP profiles before and following PNP degradation respectively (*P. syringae* highlighted in green). Dispersal visualised by decomposing the
 overall distribution using an index of dispersion based on the ratio of
 variance to the mean abundance for each bacterial TRF from analysed 9
 435 TRFLP profiles. The line depicts the 2.5% confidence limit for the χ^2
 distribution. The 97.5% confidence limit was not plotted, as no taxon fell
 below that line.

440 Analysis of slopes of rank-abundance plots (SI Table 1, Figure 4)
 revealed significant differences ($p < 0.001$) between the T_0 and T_{end}
 metacommunities. Shifts in the November and February metacommunities
 were observed with increasing slopes between T_0 and T_{end} from $b = -0.0426$ to
 $b = -0.0356$ ($p < 0.0001$) in November, and from $b = -0.0386$ to $b = -0.0221$
 445 ($p < 0.0001$) in February. This indicates a more even community structure
 after PNP biodegradation. However, in May the biofilm metacommunity
 became more heterogeneous since the slope changed between T_0 and T_{end}
 from $b = -0.0155$ to $b = -0.0177$ ($p < 0.0001$). The data indicate increased
 relative abundance of *P. syringae* following degradation of PNP in February
 450 and May biofilms, but not in those from November.

Supplement Table 1. Comparison of slopes of metacommunity rank-
 abundance plots prior to and following PNP degradation.

Rank- abundance plot	<i>F</i> -value	Slope (<i>b</i>)	<i>P</i> -value	<i>r</i> ²
November T ₀	<i>F</i> _{1,31} =315.78	-0.0426 ^a	<0.0001	0.91
November T _{end}	<i>F</i> _{1,34} =266.61	-0.0356 ^b	<0.0001	0.89
February T ₀	<i>F</i> _{1,31} = 165.96	-0.0386 ^{ac}	<0.0001	0.84
February T _{end}	<i>F</i> _{1,53} =1176.32	-0.0221 ^d	<0.0001	0.96
May T ₀	<i>F</i> _{1,79} =1362.73	-0.0155 ^e	<0.0001	0.95
May T _{end}	<i>F</i> _{1,75} =452.11	-0.0177 ^f	<0.0001	0.86

Treatments with different letters are significantly different (*P*<0.05); *n*=3.

455

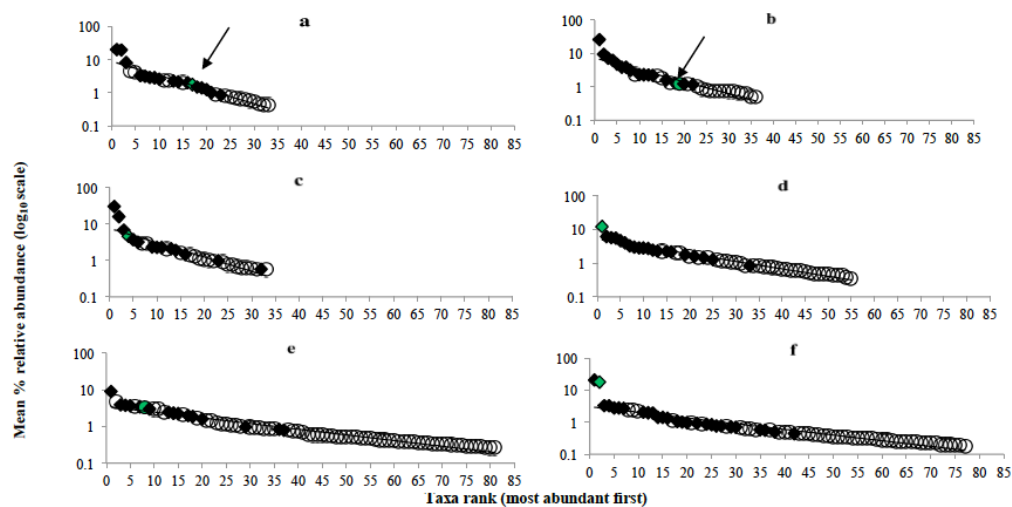


Fig 4. Rank-abundance plots of core and satellite taxa in biofilms at prior to and following PNP degradation at different sampling times

460 Figure legend: a, and b are November T_0 and T_{end} ; c, d are February T_0 and T_{end} ; e and f are May T_0 and T_{end} . T_0 , freshly collected biofilms; T_{end} , biofilms after complete PNP biodegradation. The relative rank positions of core group (closed diamonds), satellite group (open circles) and *Pseudomonas syringae* (highlighted in green) are given. Each plot has been fitted with a slope.

465

4. Discussion

In the current study we have demonstrated that the major factor determining microbial community composition of *in situ* biofilms and river water was sampling time, with minor differences between sampling locations. Biofilms
470 taken at different sampling times had different potential for chemical biodegradation, with higher biodegradability potential observed for inocula collected in February and May 2012 in comparison with inocula sampled in November 2011. Significantly, differences in PNP degradation between sampling locations were identified in November 2011, with 2 of the 3
475 locations showing no potential for biodegradation.

River biofilm communities are known to show considerable spatial and temporal variation associated with seasonal variation in water characteristics (Anderson-Glenna et al., 2008). Brümmer et al. (2000) reported clear seasonal peaks of abundance among major phylogenetic groups of bacteria
480 in a polluted river over an annual cycle. Similarly, Böckelmann et al. (2000) described a reduction of metabolic activity of microbial communities of aggregates of phototrophic and heterotrophic microorganisms in the Elbe

River, from summer (August) to winter (December) for both, free-living and surface associated bacterial populations. Seasonal changes in biofilm
485 community structure and function may have implications for chemical biodegradation. Palmisano et al. (1991) found lower first-order rate constants for 2,4D mineralization in river biofilms collected in the winter relative to the summer. Furthermore, Chénier et al. (2003) showed that hexadecane biodegradation potential of biofilm grown in bioreactors from river water
490 inoculum depended on season. River water collected in autumn generated biofilm with greater mineralization potential than material from other seasons.

Variability in the biomass and diversity of inocula used in OECD biodegradability tests has been identified as the main reason for test failures and the inconsistency of test results (Courtes et al. 1995; Mezzanotte et al.
495 2005). Efforts have been made to standardize microbial inocula prior to biodegradation testing (e.g. increasing microbial biomass via filtration or centrifugation) and several pre-adaptation strategies have been developed (e.g. semi-continuous pre-exposure procedure) (Toräng and Nyholm, 2005). Although increases in inoculum biomass and pre-adaptation of inocula is
500 known to increase the probability of chemical degradation, inoculation with high amounts of microbial biomass may still fail to degrade the chemical (Kool, 1984; Szabó et al. 2007; Thouand et al. 2011).

Com

A 'biodegradation lottery' has been suggested by several authors as the
505 main reason for test failures, due to uneven distribution of specific degraders within aliquots of inoculum used in biodegradation tests (Thouand et al. 2011). Alternatively, microbe-microbe interactions which prevent proliferation

of a degrader could also contribute to test failures. In our study, *pnpA* genes, which encode 4-nitrophenol 4 monooxygenase, the first enzyme in the Gram-negative pathway for PNP degradation, were found in all samples, including
510 those from November 2011, in which no degradation of PNP was detected. *P. syringae* was previously reported by (Kowalczyk et al. 2015) as a PNP degrader. Sequencing of both 16S rRNA genes and *pnpA* genes indicated that PNP degraders present in river water samples belong to *Pseudomonas*
515 spp. Similarly, community analysis indicated that a ribotype indicative of *P. syringae*, identified as the key PNP degrader in samples from this river in earlier work (Kowalczyk et al. 2015) was present in all samples irrespective of whether they subsequently degraded PNP.

Test failure arising from a biodegradation lottery because of an uneven
520 distribution of degraders between biodegradation tests would be indicative of degraders showing low abundance and persistence, and thereby being members of the satellite community. However, our results show that the *P. syringae* ribotype was a satellite taxon in February 2012, yet proliferated across all samples following PNP degradation to become the most abundant
525 taxon. In contrast, in November 2011 and May 2012, the *P. syringae* ribotype was a core component of the community, yet it only proliferated in the May samples, with degradation rates across all locations similar to the February samples. However, despite being a core community component in the November 2011 samples, its taxa rank was 18, compared to 1 and 2 in the
530 February and May 2012 samples, respectively.

Differences in biodegradation of PNP by biofilms at different time points could be due to a variety of mechanisms. Failure of PNP degraders to

proliferate could result from microbe-microbe interactions, which may be competitive or synergistic. For example, the persistence of specific
535 degraders in the community may require the presence of other bacterial species to provide cofactors required for growth (Bending et al., 2003). Therefore, microbial interactions within inoculum could determine the proliferation of specific degraders in microbial populations thereby impacting the outcome of biodegradation tests. McGenity et al. (2012) reported that a
540 network of direct and indirect interactions within and between species is observed in the presence of hydrocarbons in marine environment. Some of the key cooperative interactions may include consumption of metabolites, biosurfactant production, provision of oxygen and fixed nitrogen. Microbial consortia are known to have better ability to degrade complex pollutants than
545 single strains (Hoskeri et al. 2014; Rahman et al. 2002). Greater resolution of the biofilm composition and organisation such as studying matrix composition, viable cell number and localization of degraders (Pantarella et al. 2013) could help explain seasonal differences in biodegradation potential.

However, in the case of degradation of PNP, which is typically a readily
550 degraded chemical, no such synergisms have been identified (Kowalczyk et al. 2015), suggesting that competitive interactions could have contributed to test failures.

Approaches to profile complex microbial communities continue to evolve, and metagenomic, metaproteomic and metatranscriptomic tools are
555 becoming increasingly accessible. They allow detection of unculturable microbes and link their presence to environmental processes, including

chemical degradation, without biases associated with PCR primers (Fang et al. 2013; Kowalczyk et al. 2015).

In conclusion, river collected biofilms showed considerable seasonal
560 variation in biomass, microbial community composition and PNP
biodegradation potential, and there was also evidence that location at which
the biofilm was grown affected biodegradation outcome. These
characteristics may limit the usefulness of field grown biofilms as inocula for
biodegradation tests. However, our data show that biodegradation test
565 failures could arise from microbe-microbe interactions rather than a failure of
inocula to contain specific degraders or genetic potential for biodegradation.

Acknowledgements

We thank the Safety and Environmental Assurance Centre, UK, Unilever for
570 the financial support of this study.

References

- Ager, D., Evans, S., Li, H., Lilley, A.K., van der Gast, C.J., 2010. Anthropogenic disturbance affects the structure of bacterial communities. Environ. Microbiol., 12, 670-678.
- Bending, G. D., Lincoln, S.D., Sørensen, S.R., Morgan, J.A.W., Aamand, J. Walker, A. 2003. In-field spatial variability in the degradation of the phenyl-urea herbicide isoproturon is the result of interactions between degradative *Sphingomonas* spp. and soil pH. Appl. Environ. Microbiol. 69, 827-834.
- Bertram, D.F., Mackas, D.L., McKinnell, S.M., 2001. The seasonal cycle revisited: interannual variation and ecosystem consequences. Prog. Oceanogr. 49: 283-307.
- Böckelmann, U., Manz, W., Neu, T.R., Szewzyk, U., 2000. Characterization of the microbial community of lotic organic aggregates ('river snow') in the Elbe River of Germany by cultivation and molecular methods. FEMS Microbiol. Ecol. 33, 157-170.
- Brümmer, I.H.M., Fehr, W., Wagner-Döbler, I., 2000. Biofilm community structure in polluted rivers: abundance of dominant phylogenetic groups over a complete annual cycle. Appl. Environ. Microbiol. 66, 3078-3082.
- Cébron, A., Coci, M., Garnier, J., Laanbroek, H.J., 2007. Denaturing gradient gel electrophoretic analysis of ammonia-oxidizing bacterial community structure in the lower Seine River: impact of Paris wastewater effluents. Appl. Environ. Microbiol. 70, 6726-6737..
- Chénier, M.R., Beaumier, D., Roy, R., Driscoll, B.T., Lawrence, J.R., Greer, C.W., 2003. Impact of seasonal variations and nutrient inputs on nitrogen

cycling and degradation of hexadecane by replicated river biofilms. *Appl. Environ. Microbiol.* 69, 5170-5177.

Courtes, R., Bahlaoui, A., Rambaud, A., Deschamps, F., Sunde, E.,
600 Dutrieux, E., 1995. Ready biodegradability test in seawater: a new
methodological approach. *Ecotox. Environ. Safe.* 31, 142-148.

Deines, P., Sekar, R., Husband, P.S., Boxall, J.B., Osborn, A.M. and Biggs,
C.A., 2010. A new coupon design for simultaneous analysis of in situ
microbial biofilm formation and community structure in drinking water
605 distribution systems. *Appl. Microbiol. Biotechnol.*, 87, 749-756.

Doi, Y., Kasuya, K., Abe, H., Koyama, N., Ishiwatari, S., Takagi, K., Yoshida,
Y., 1996. Evaluation of biodegradabilities of biosynthetic and
chemosynthetic polyesters in river water. *Pol. Deg. Stab.* 51, 281-286.

Donlan, R.M. 2002. Biofilms: microbial life on surfaces. *Emerg Infect Dis.* 8,
610 881-890.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956.
Colorimetric method for determination of sugars and related substances.
Anal. Chem. 28, 350–356.

Elias, S., Banin, E., 2012. Multi-species biofilms: living with friendly
615 neighbors. *FEMS Microbiol. Rev.* 35, 990-1004.

Fang, H., Cai, L., Yu, Y., Zhang, T., 2013. Metagenomic analysis reveals the
prevalence of biodegradation genes for organic pollutants in activated
sludge. *Biores. Technol.* 129, 209-218.

Flemming H.-C., Neu, T.R., Wozniak, D.J., 2007. The EPS matrix: The
620 “House of Biofilm Cells”. *J. Bacteriol.* 189, 7945-7947.

Godhead, A.K., Head, I.M., Snape, J.R., Davenport, R.J., 2014. Standard

- inocula preparations reduce the bacterial diversity and reliability of regulatory biodegradation tests. *Environ. Sci. Pollut. Res.* 21, 9511-9521.
- Hilton, S., Bennett, A.J., Keane, G., Bending, G.D., Chandler, D., Stobart, R.,
625 Mills, P., 2013. Impact of shortened crop rotation of oilseed rape on soil and rhizosphere microbial diversity in relation to yield decline. *PLOS ONE* 8, 1-12.
- Hoskeri, R.S., Mulla, S.I., Ninnekar, H.Z., 2014. Biodegradation of chloroaromatic pollutants by bacterial consortium immobilized in
630 polyurethane foam and other matrices. *Biocat. Agri. Biotechnol.* 3, 390-396.
- Hudon, C., 1997. Impact of water level fluctuations on St. Lawrence River aquatic vegetation. *Can. J. Fish. Aquat. Sci.* 54, 2853-2865.
- Hunt, A.P., Parry, J.D., 1998. The effect of substratum roughness and river
635 flow rate on the development of a freshwater biofilm community. *Biofouling* 12, 287-303.
- Jones, O.A.H., Voulvoulis, N., Lester, J.N., 2003. Potential impact of pharmaceuticals on environmental health. *Bull. World. Health. Organ.* 81.
<http://dx.doi.org/10.1590/S0042-96862003001000015>
- 640 Kargi, F., Eker, S., 2005. Removal of 2,4-dichlorophenol and toxicity from synthetic wastewater in a rotating perforated tube biofilm reactor. *Proc. Biochem.* 40, 2105-2111.
- Kool, H.J. 1984. Influence of microbial biomass on the biodegradability of organic compounds. *Chemosphere* 7:751-761.
- 645 Kowalczyk, A., Martin, T.J., Price, O.R., Snape, J.R., van Egmond, R.A., Finnegan, C.J., Schäfer, H., Devenport, R.J., Bending, G.D., 2014.

- Refinement of biodegradation tests methodologies and the proposed utility of new microbial ecology techniques. *Ecotox. Environ. Safety*. 111, 9-22.
- Kowalczyk, A., Eyice, Ö., Schäfer, H., Price, O.R., Finnegan, C.J., van Egmond, R.A., Shaw, L.J., Barrett, G. and Bending, G.D. 2015. Characterization of *para*-nitrophenol degrading bacterial communities in river water using functional markers and stable isotope probing. *Appl. Environ. Microbiol.* doi: 10.1128/AEM.01794-15.
- Krebs, C.J., 1999. *Ecological Methodology* 2nd edn. Harper and Row: New York, NY.
- LaPara, T.M., Konopka, A., Nakatsu, C.H., Alleman, J.E., 2000. Effects of elevated temperature on bacterial community structure and function in bioreactors treating a synthetic wastewater. *J. Ind. Microbiol. Biotech.* 24, 140-145.
- Lapertot, M.E., Pulgarin, C., 2006. Biodegradability assessment of several priority hazardous substances: Choice, application and relevance regarding toxicity and bacterial activity. *Chemosphere*, 65, 682-690.
- Mah, T.F.C., O'Toole, G.A., 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol.* 9, 34-39.
- McGenity, T., Folwell, D., McKew, B.A., Sanni, G.O., 2012. Marine crude-oil biodegradation: a central role for interspecies interactions. *Aquat. Biosys.* 8,10.
- Mezzanotte, V., Bertani, R., Innocenti, F.D., Tosin, M., 2005. Influence of inocula on the results of biodegradation tests. *Polym. Deg. Stab.* 87, 51-56.
- Midwood, J.D., Chow-Fraser, P., 2012. Changes in aquatic vegetation and

- fish communities following 5 years of sustained low water levels in coastal marshes of eastern Georgian Bay, Lake Huron. *Global Change Biol.* 18, 93-105.
- 675 Mnif, W., Hassine, A.I.H., Bouaziz, A., Bartegi, A., Thomas, O., Roig, B., 2011. Effect of endocrine disruptor pesticides: a review. *Int. J. Environ. Res. Public Health.* 8, 2265–2303.
- Morin, S., Pesce, S., Tlili, A., Coste, M., Montuelle, B., 2007. Recovery potential of periphytic communities in a river impacted by a vineyard
- 680 watershed. *Ecol. Indicators* 10, 419-426.
- Naudin, J.J., Cauwet, G., Fajon, C., Oriol, L., Terzić, S., Devenon, J.L., Broche, P., 2001. Effect of mixing on microbial communities in the Rhone River plume. *J. Marine Sys.* 28, 203-227.
- Nesbit, H.J., Watson, J.R., 1980. Degradation of the herbicide 2,4-D in river
- 685 water-II. The role of suspended sediment, nutrients and water temperature. *Wat. Res.* 14, 1689-1694.
- OECD Guideline for testing of chemicals, 2005. Proposal for revised introduction to the OECD Guidelines for testing of chemicals, section 3. Annex 1. ENV/JM/TG(2005)5/REV1.
- 690 Oh, K.H., Tuovinen, O.H., 1994. Biodegradation of the phenoxy herbicides MCPP and 2,4-D in fixed-film column reactors. *Int. Biodet. Biodeg.* 33, 93-99.
- Palmisano, A.C., Schwab, B.S., Maruscik, D.A., Ventullo, M.R., 1991. Seasonal changes in mineralization of xenobiotics by stream microbial
- 695 communities. *Can. J. Microbiol.* 37, 939-948.
- Pantanella, F., Valenti, P., Natalizi, T., Passeri, D., Berlutti, F., 2013.

- Analytical techniques to study microbial biofilm on abiotic surfaces: pros and cons of the main techniques currently in use. *Ann. Ig.* 25, 31-42.
- Proia, L., Morin, S., Peipoch, M., Romaní, A.M., Sabater, S., 2010.
 700 Resistance and recovery of river biofilms receiving short pulses of triclosan and diuron. *Sci. Tot. Environ.* 409, 3129-3137.
- Puhakka, J.A., Herwig, R.P., Koro, P.M., Wolfe, G.V., Ferguson, J.F., 1995.
 Biodegradation of chlorophenols by mixed and pure cultures from a fluidized-bed reactor. *Appl. Micro. Biotech.* 42, 951-957.
- 705 Rahman, K.S.M., Thahira-Rahman, J., Lakshmanaperumalsamy P., Banat I.M., 2002. Towards efficient crude oil degradation by a mixed bacterial consortium. *Biores. Technol.* 85, 257-261.
- Reuschenbach, P., Pagga, U., Strotmann, U., 2003. A critical comparison of respirometric biodegradation tests based on OECD 301 and related test
 710 methods. *Wat. Res.* 37, 1571-1582.
- Rodriguez-Cruz, M.S., Jones, J.E., Bending, G.D., 2006. Field-scale study of the variability in pesticide biodegradation with soil depth and its relationship with soil characteristics. *Soil. Biol. Biochem.* 38, 2910-2918.
- Rogers, G.B., Cuthbertson, L., Hoffman, L.R., Wing, P.A.C., Pope, C.,
 715 Hooftman, D.A.P., Lilley, A.K., Oliver, A., Carroll, M.P., Bruce, K.D. and van der Gast, C.J., 2013. Reducing bias in bacterial community analysis of lower respiratory infections. *The ISME Journal*, 7, 697-706.
- Romaní, A.M., Sabater, S., 1999. Effect of primary producers on the heterotrophic metabolism of a stream biofilm. *Freshw. Biol.* 41, 729-736.
- 720 Sabater, S., Guasch, H., Ricart, M., Romaní, A., Vidal, G., Klünder, C., Schmitt-Jansen, M., 2007. Monitoring the effect of chemicals on biological

- communities. The biofilm as an interface. *Anal. Bioanal. Chem.* 387, 1425-1434.
- Schwartz, T., Kohnen, W., Jansen, B., Obst, U., 2003. Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS Microbiol. Ecol.* DOI: <http://dx.doi.org/10.1111/j.1574-6941.2003.tb01073.x> 325-335.
- Singh, R., Paul, D., Jain, R.K., 2006. Biofilms: implications in bioremediation. *Trends Microbiol.* 14, 389-397.
- 725 Szabó, K.E., Itor, P.O.B., Bertilsson, S., Tranvik, L., Eiler, A., 2007. Importance of rare and abundant population for the structure and functional potential of freshwater bacterial communities. *Aquatic Microbial Ecol.* 47,1-10.
- Thouand, G., Durand, M.J., Gancet, C., Blok, H., 2011. New concepts in the evaluation of biodegradation/persistence of chemical substances using a microbial inoculum. *Front. Microbiol.* 2, 1-6.
- 735 Thouand, G., Friant, P., Bois, F., Cartier, A., Maul, A., and Block, J.C., 1995. Bacterial inoculum density and probability of para-nitrophenol biodegradability test response. *Ecotox. Environ. Safe.* 30, 274-282.
- 740 Toräng, L., and Nyholm, N., 2005. Biodegradation rates in adapted surface water can be assessed following a preadaptation period with semi-continuous operation. *Chemosphere* 61, 1-10.
- Underwood, G.J.C., Paterson, D.M., 1995. The measurement of microbial carbohydrate exopolymers from intertidal sediments. *Limnol. Oceanogr.* 40, 1243-1253.
- 745 van der Gast, C.J., Walker, A.W., Stressmann, F.A., Rogers, G.B., Scott, P.,

- Daniels, T.W., Carroll, M.P., Parkhill, J., Bruce, K.D., 2011. Partitioning core and satellite taxa from within cystic fibrosis lung bacterial communities. *The ISME J.* 5, 780-791.
- 750 Watson, J.R., 1977. Seasonal-variation in biodegradation of 2,4-D in river water. *Wat. Res.*, 11, 153-157.
- Yuan, S.Y., Yu, C.H., Chang, B.V., 2004. Biodegradation of nonylphenol in river sediment. *Environ. Poll.* 127, 425-430.
- Yunus, A.J.M., Nakagoshi, N., 2004. Effects of seasonality on streamflow
755 and water quality of the Pinang River in Penang Island, Malaysia. *Chinese Geogr. Sci.* 14, 153-161.
- Zhang, T.C., Fu, Y.C., Bishop, P.L., Kupferle, M., FitzGerald, S., Jiang, H.H., Harmer, C., 1995. Transport and biodegradation of toxic organics in

760